Scale Up with Confidence

Column selection for preparative HPLC

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Columns and Supplies Technical Support
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Agilent InfinityLab

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What is Preparative LC and Why Do We Do It?

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Goal of preparative LC

- Collect one or more compounds from a sample

Differences Between Analytical and Preparative LC

	Analytical LC	Preparative LC
Objective	**Quantitation** – develop a reproducible method to quantify an analyte. Complete separation isn't required (with certain detectors or integration techniques)	**Purification** – purify analyte from rest of matrix. Coeluting impurities will impact purity of collection and could impact downstream testing results
Environment	**GLP/GMP** – prescriptive rules for method development; once method is validated for an analyte, very little deviation is allowed	**Discovery** – analysis of novel compounds; no precedent; a lot of flexibility around column choice and run conditions; chemist can make changes on the fly
Peak Shape	**Gaussian** – concentration levels must be within linear range of detector	**Non-Gaussian** – concentration levels can overload the column and saturate the detector
Priority	Reproducible results – some injection modes can waste sample	Minimum sample loss – injection reproducibility doesn't matter

Small molecule purification environments

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High throughput

Objectives

- Purifying many different samples
- Small amounts of material for preliminary bioactivity testing or further characterization
- Target – main component

Workflow

- Not much time can be spent on method development – analytical screen to determine best column, then utilize focused gradient to improve separation (if necessary)
- Requires small amounts of many different samples, typically 1-2 injections per sample

Applications

- Combinatorial chemistry libraries
- Reaction cleanup
- Open access purification for medicinal chemists

Bulk purification

Objectives

- Purifying one sample
- Significant amounts of material for more in-depth testing and characterization
- Target – main component or an impurity

Workflow

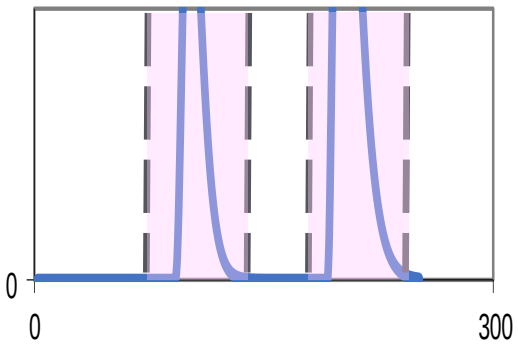
- Considerable time is spent optimizing method to maximize yield, purity and minimize solvent consumption
- Many injections of the same sample

Applications

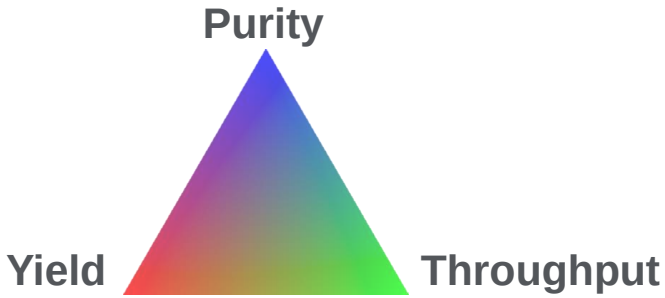
- Purification of target compounds (API), Natural products, etc..
- Impurity isolation for Identification, structure elucidation and activity testing

Objectives of Preparative LC: purity, yield and throughput

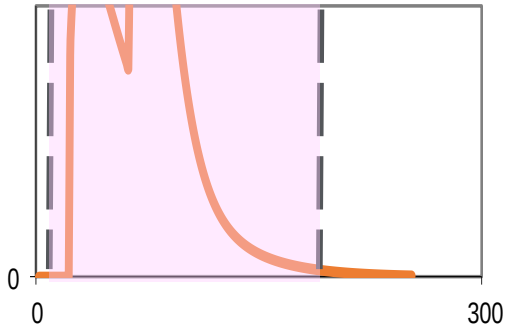
- **Purity**
- **Yield**
- **Throughput**



Longer run times to have enough separation between peaks



- **Throughput**
- **Yield**
- **Purity**

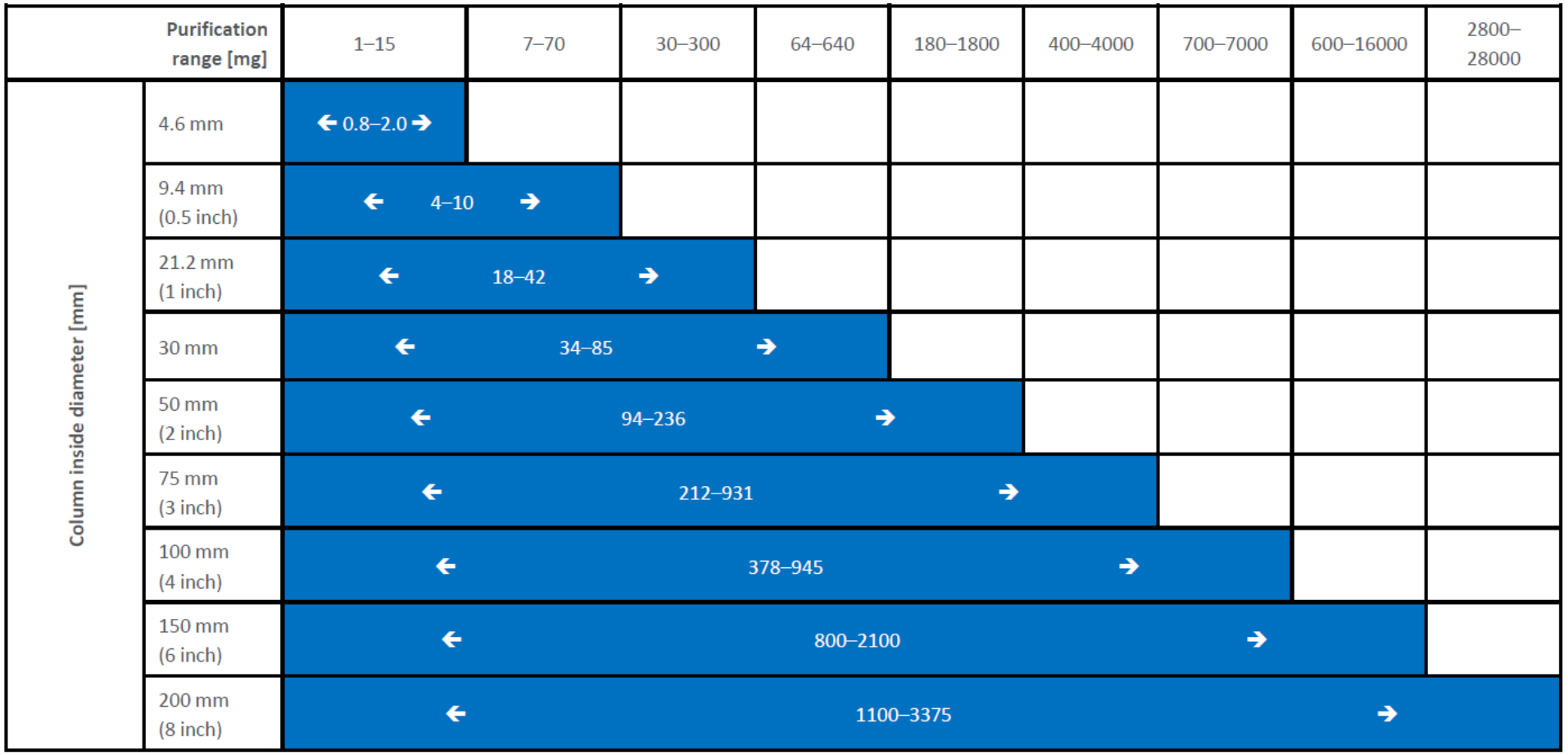


Shorter run times, but collected fractions may contain impurities

You can optimize two of the parameters by sacrificing the third.
The most important parameter depends on the application.

Column Dimensions, Flow Rates and Column Loads

Choosing the right parameters

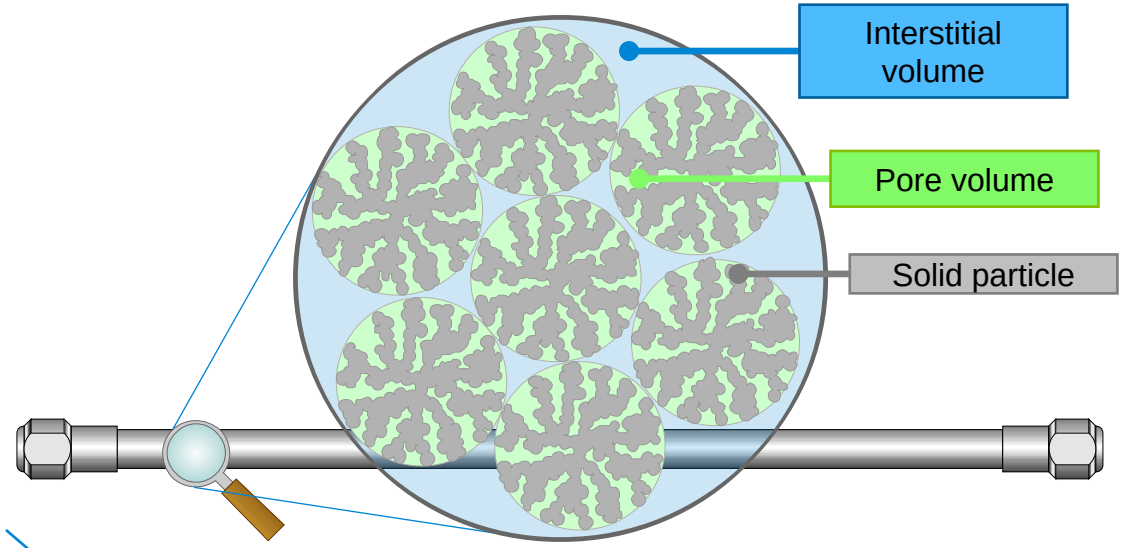


InfinityLab Preparative HPLC Columns

Family	Phase	21.2 mm ID				30 mm ID			
		50	100	150	250	50	100	150	250
Poroshell	SB-C18	670050-902	--	670150-902	--	--	--	--	--
	HPH-C18	670050-702	--	670150-702	--	--	--	--	--
ZORBAX	Eclipse Plus C18	595050-902	595100-902	595150-902	595250-902	575050-902	575100-902	575150-902	575250-902
	Eclipse Plus C8	595050-906	595100-906	595150-906	595250-906	575050-906	575100-906	575150-906	575250-906
	SB-C18	585050-902	585100-902	585150-902	585250-902	565050-902	565100-902	565150-902	565250-902
	SB-C8	585050-906	585100-906	585150-906	585250-906	565050-906	565100-906	565150-906	565250-906
	EP Phenyl-Hexyl	595050-912	595100-912	595150-912	595250-912	575050-912	575100-912	575150-912	575250-912
Pursuit XRs	C18	INF6000050X212	INF6000100X212	INF6000150X212	INF6000250X212	INF6000050X300	INF6000100X300	INF6000150X300	INF6000250X300
	C8	INF6010050X212	INF6010100X212	INF6010150X212	INF6010250X212	INF6010050X300	INF6010100X300	INF6010150X300	INF6010250X300
	Diphenyl	INF6020050X212	INF6020100X212	INF6020150X212	INF6020250X212	INF6020050X300	INF6020100X300	INF6020150X300	INF6020250X300

Sample Loading and Surface Area

Surface area: Refers to the total area of the solid surface on the HPLC particle. The surface area of a typical porous adsorbent such as silica gel can vary from less than 100 to 600 m^2/g.



Phase	Surface Area (m^2/g)	Pore Size (Å)
Poroshell SB-C18 (4 µm SPP)	130	120
Pursuit XRs C18	440	100

Higher surface area phases are ideal for loading

- Lower surface area phases offer fast separations
- Smaller particle sizes offer higher efficiency to resolve complicated matrices

Agilent Biomolecule Columns Portfolio

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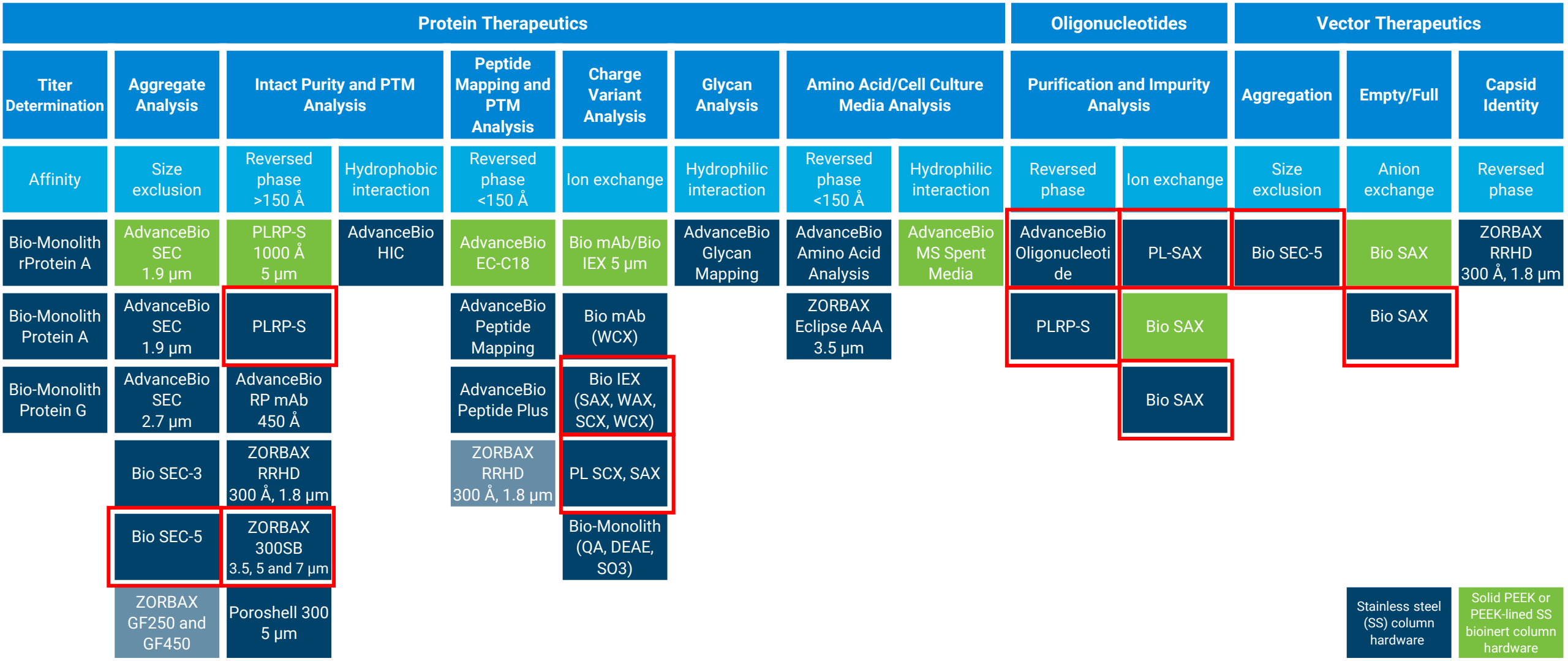

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Flow Rate (Biomolecules)

The optimum flow rate for reversed phase and ion exchange preparative LC columns is 360 cm/h or less (equivalent to 1 mL/min on a 4.6 mm id column).

Flow rates equivalent to 360 cm/h suitable for reversed phase and ion exchange columns

4.6 mm ID	10 mm ID	21.2 mm ID	30 mm ID	50 mm ID
1.0 mL/min	4.7 mL/min	21.2 mL/min	42.5 mL/min	118 mL/min

The optimum flow rate for size exclusion columns is 120 cm/h or less (equivalent to 1 mL/min on a 7.8 mm id column).

Flow rates equivalent to 120 cm/h suitable for size exclusion columns

7.8 mm ID	10 mm ID	21.2 mm ID	30 mm ID	50 mm ID
1.0 mL/min	1.6 mL/min	7.4 mL/min	15 mL/min	41 mL/min

Steps in Developing a Preparative Method

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1. Check sample formulation	2. Screen columns	3. Optimize the separation	4. Determine maximum sample load	5. Determine the preparative column size	6. Scale method to preparative column	7. Purify the compound(s)
Solvent, concentration, additives	Run analytical (scalar) columns	Maximize the resolution between the target compound and nearest peaks	Calculate resolution as a function of mass and volume loading	Use the analytical column loading data	Use linear scale up factor	Measure purity and amount of compounds obtained
Confirm LC method compatibility	Select optimum column phase	Confirm the conditions compatible with compound stability/next step	Optimum loading to achieve objectives	Confirm compatible with preparative instrument	Confirm compatible with preparative instrument	Confirm objectives met

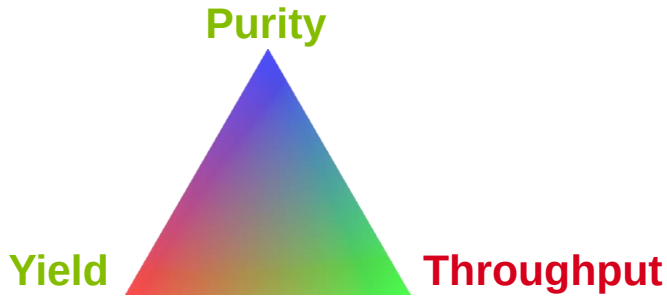
Bulk Purification

Objectives

- A colleague has synthesized a proprietary small molecule.
- They would like to have their target compound purified from their crude mix (1 g material) for additional characterization.
- The collaborator has developed an analytical method on a C18 column that is not available in a preparative dimension.

Priority checklist

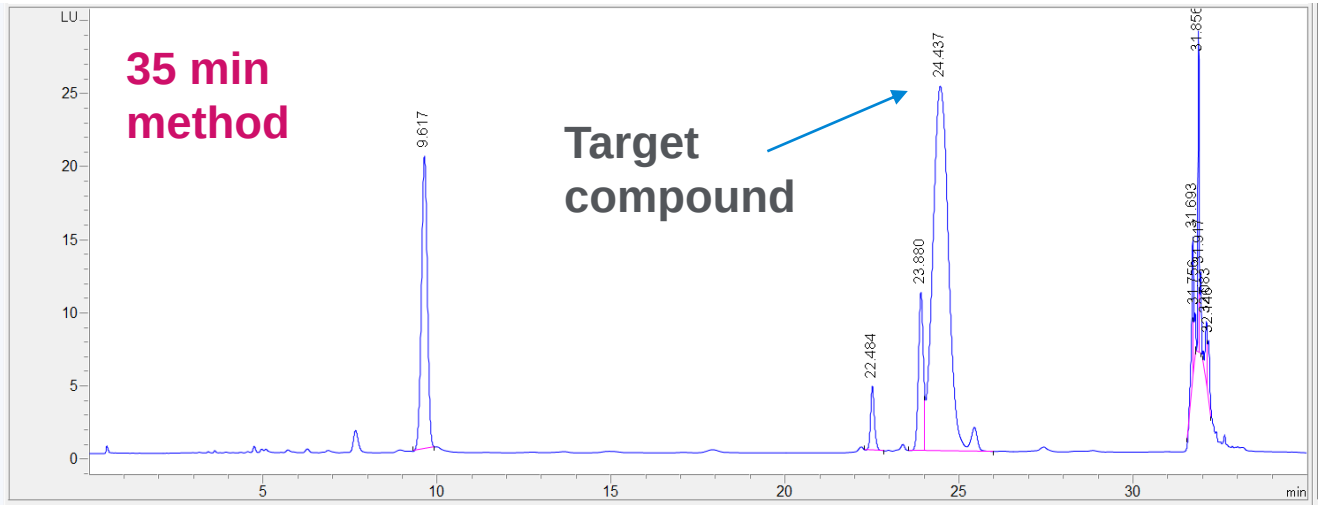
- Purity?
- Yield?
- Throughput?



Fits bulk purification

Analyzing the Analytical Method

- Method used to confirm synthesized product
- Column: C18, 4.6 x 150 mm, 5 µm
- Injection volume: 1 µL
- Mobile phase:
  - A: H_2O + 0.1% formic acid
  - B: ACN + 0.1% formic acid
- Gradient:
  - 5% B for 0.5 min
  - 10% B for 19.5 min
  - 18% B for 10 min
  - 80% B flush for 3 min
  - 5% B for 2 min



How can this be converted into a successful purification method?

- Develop on an analytical phase available in preparative dimensions
- Improve separation between product and impurities
- Increase the sample load on the column
- Reduce run time

Step 1: Check Sample Formulation and Solubility

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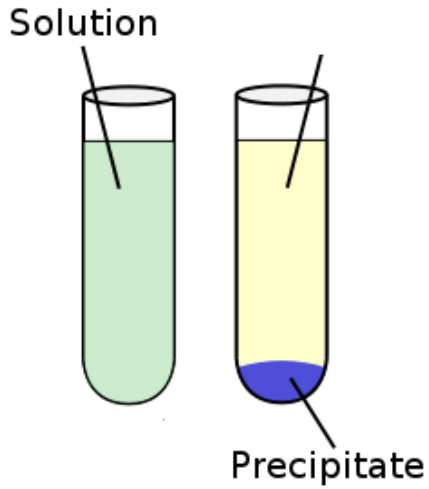
1. Check sample formulation | 2. Screen columns | 3. Optimize separation | 4. Determine maximum sample load | 5. Determine prep column size | 6. Scale conditions to prep column | 7. Purify the compounds

- **Verify sample is compatible with reversed-phase LC**
  - Collaborator has had success with C18 phase

- **Verify sample is soluble in mobile-phase compatible solvent**
  - Acetonitrile, methanol, ethanol, water
  - Many samples are dissolved in a strong solvent (DMSO, DMF)
  - Low solubility in mobile phase = sample precipitating (crashing) out of solution
  - Strong solvents can impact the peak shape and loading of early eluting peaks



- Sample provided as a dried powder
- Soluble up to 20 mg/mL in 50:50 ethanol:water

Test solubility by putting a few drops of sample solution into a beaker of mobile phase (starting composition)

Step 2: Screen Columns and Mobile Phases

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1. Check sample formulation | **2. Screen columns** | 3. Optimize separation | 4. Determine max sample load | 5. Determine prep column size | 6. Scale conditions to prep column | 7. Purify the compounds

Column chemistry and mobile phase have the biggest impact on separation

If you are developing a method for a new/unknown compound:

- Screen different column/mobile phase combinations with a generic gradient (5-95%)
- Columns: screen several columns with complementary selectivities
- Mobile phase: water:acetonitrile and water:methanol are most common
- pH: If the pH ≈ the compound's pK (dissociation constant), bad peak shape or split peak
  - Operate at a pH that is 1-2 units away from the pKa

If you already have a method:

- Make sure there is a matching preparative column

Make sure you have both analytical and preparative columns that are the same phase

Step 2: Screening Study

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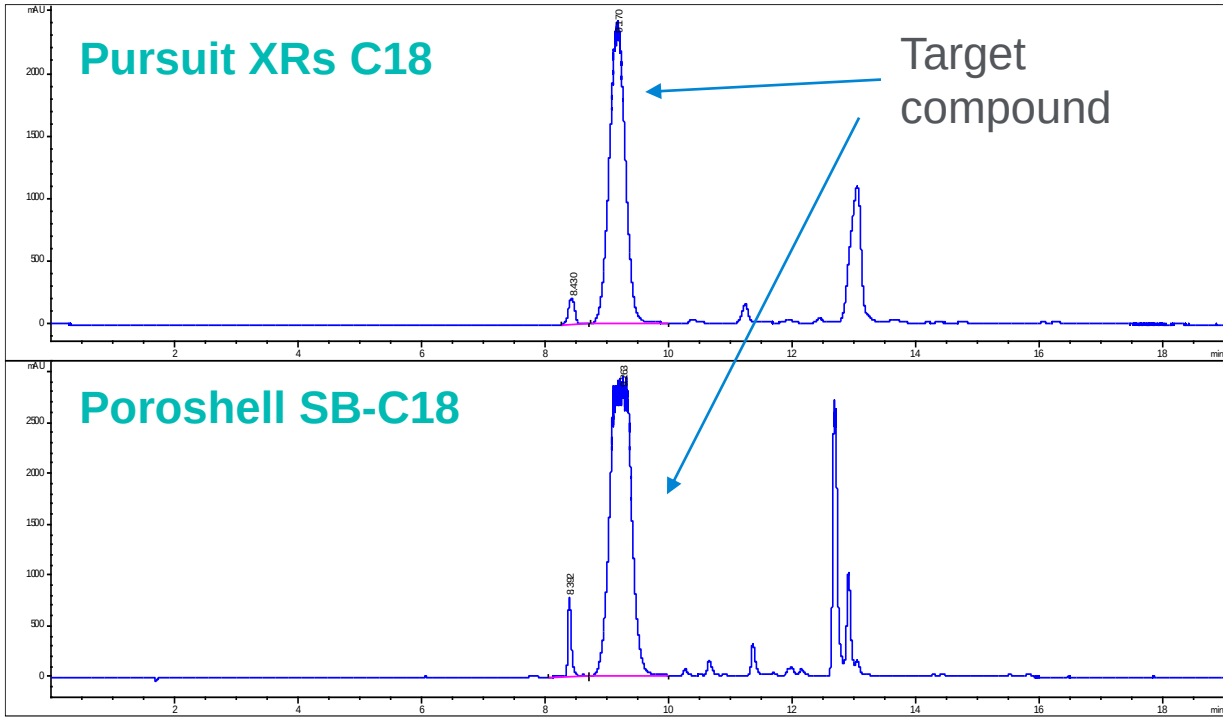
1. Check sample formulation | **2. Screen columns** | 3. Optimize separation | 4. Determine max sample load | 5. Determine prep column size | 6. Scale conditions to prep column | 7. Purify the compounds

Modified screening study

- Collaborator's method run on C18
- Columns (available in preparative dimensions)
  - Agilent InfinityLab Pursuit XRs C18, 4.6 x 150 mm, 5 µm
  - Agilent InfinityLab Poroshell SB-C18, 3.0 x 150 mm, 4 µm
- Mobile phase:
  - A: H_2O + 0.1% formic acid
  - B: ACN + 0.1% formic acid
- Gradient:
  - 5 – 95% in 15 minutes
  - 95% for 3 minutes



Flow rate: XRs C18 – 1 mL/min; SB-C18 – 0.5 mL/min; mobile phase: A – water + 0.1% formic acid; B – acetonitrile + 0.1% formic acid; gradient: 5 – 95% B in 15 min, 95% B for 3 min; injection volume: XRs C18 – 20 µL; SB-C18 – 10 µL; detection: UV 335 nm.

Step 2: Screening Study

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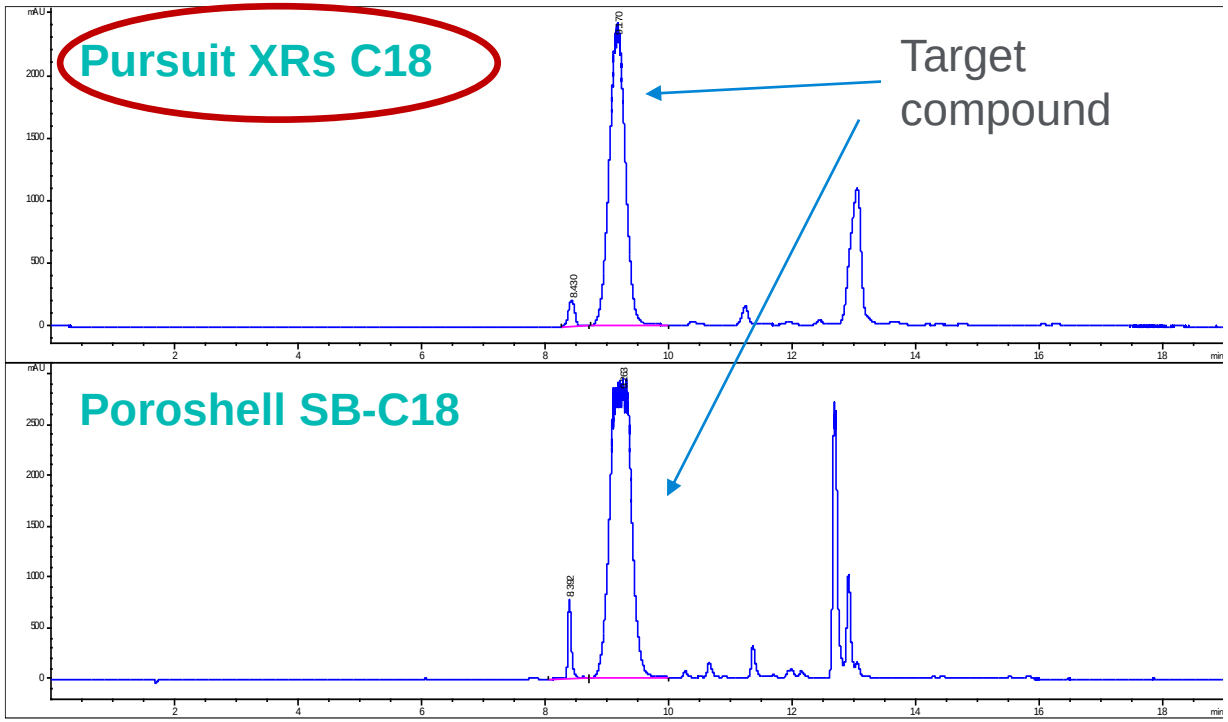
1. Check sample formulation
2. Screen columns
3. Optimize separation
4. Determine max sample load
5. Determine prep column size
6. Scale conditions to prep column
7. Purify the compounds

Results

- Both columns have similar elution; SB-C18 provides better separation for later peaks
- Focus on the critical pair – target compound and close eluting impurities
- Pursuit XRs available in 30 mm id; chosen for purification work



Flow rate: XRs C18 – 1 mL/min; SB-C18 – 0.5 mL/min; mobile phase: A – water + 0.1% formic acid; B – acetonitrile + 0.1% formic acid; gradient: 5 – 95% B in 15 min, 95% B for 3 min; injection volume: XRs C18 – 20 µL; SB-C18 – 10 µL; detection: UV 335 nm.

Step 3: Optimize the Separation of the Critical Pair

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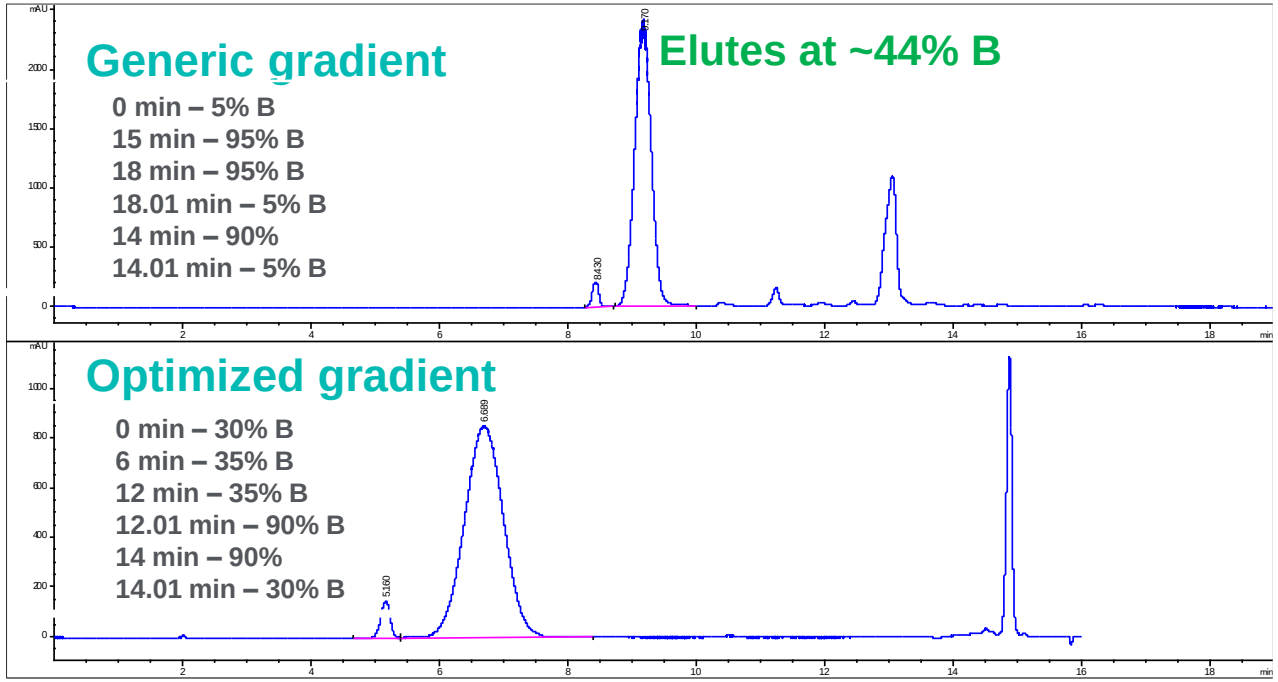
1. Check sample formulation
2. Screen columns
3. Optimize separation
4. Determine max sample load
5. Determine prep column size
6. Scale conditions to prep column
7. Purify the compounds

Things to keep in mind:

- Optimizing the method on the analytical system saves sample and solvent
- Focus on the separation of the critical pair; impurities can coelute
- Increasing the resolution of the critical pair will increase sample loading capacity

Results

- Original analytical method – 35 minutes, with partially coeluting impurities
- Optimized method – 16 minutes, with target well separated from nearest impurity



Step 4: Determine Max Sample Load

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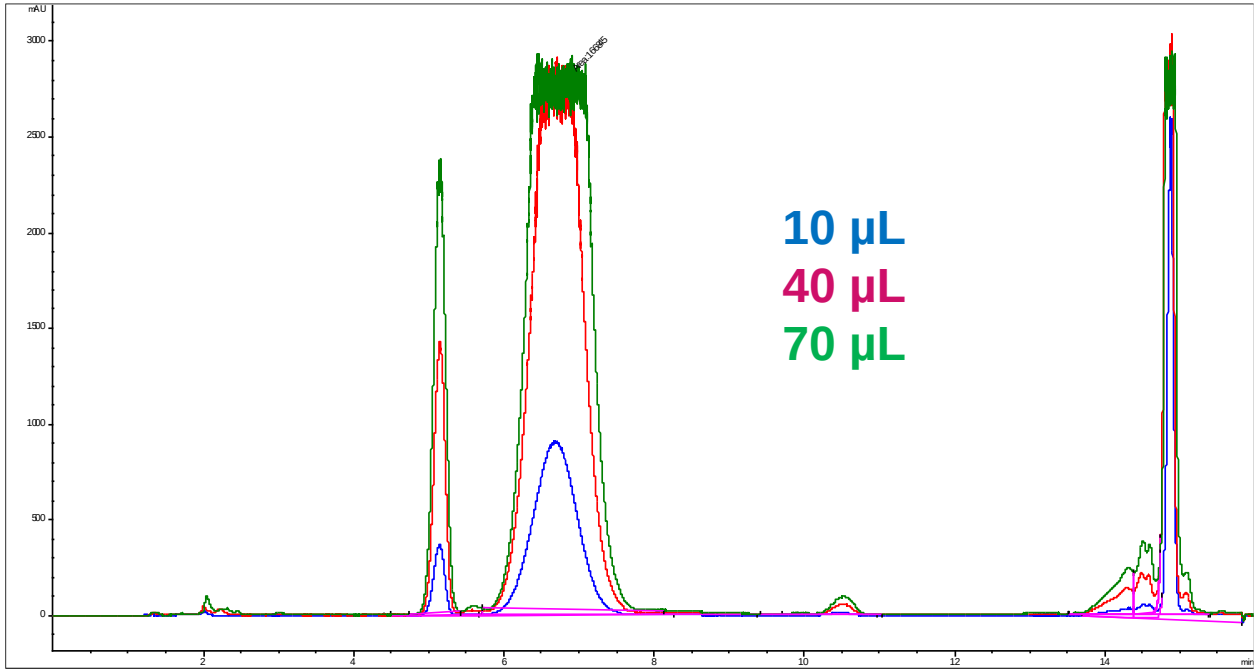
1. Check sample formulation | 2. Screen columns | 3. Optimize separation | **4. Determine max sample load** | 5. Determine prep column size | 6. Scale conditions to prep column | 7. Purify the compounds

- Injected 20 mg/mL at three injection volumes
  - 10, 40 and 70 µL
  - The largest volume (70 µL) scales to capacity of preparative sample loop (3,000 µL)
- Use the largest injection volume that still maintains separation between peaks



Results

- All three injection volumes still provide separation between the impurity and target
- 70 µL injection will be scaled to preparative instrument

It's ok if the signal is saturated. Focus on the separation at the baseline – if the peaks are separated, you're good.

Step 5: Determine Preparative Column Size

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1. Check sample formulation | 2. Screen column phases | 3. Optimize separation | 4. Determine max sample load | **5. Determine prep column size** | 6. Scale conditions to prep column | 7. Purify the compounds

- **Request:** Purify 1 g of material

General Guidelines				
Column id	Flow rate	Recommended injection volume	Easy separation (α >1.5)	Difficult separation (1.2 < α < 1.5)
4.6 mm	1 mL/min	10 µL	3 – 15 mg	0.5 – 3 mg
21.2 mm	20 mL/min	500 µL	70 – 400 mg	20 – 70 mg
30 mm	40 mL/min	1000 µL	140 – 800 mg	40 – 140 mg
50 mm	100 mL/min	2500 µL	400 – 2000 mg	100 – 400 mg

Your mileage my vary

Analytical and preparative columns that have matching phase, particle size, and length provide most consistent results.

Larger column ids can handle larger injection volumes. These are good for purifying dilute samples in fewer injections.

Step 6: Scale Conditions to Preparative Column

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1. Check sample formulation | 2. Screen columns | 3. Optimize separation | 4. Determine max sample load | 5. Determine prep column size | **6. Scale conditions to prep column** | 7. Purify the compounds

Preparative flow rate

$$f_p = f_a \left(\frac{d_p}{d_a}\right)^2$$

Where:

f_a = Flow rate of analytical column

d_p = Internal diameter of preparative column

d_a = Internal diameter of analytical column

Preparative injection volume

$$V_p = V_a \left(\frac{d_p}{d_a}\right)^2$$

Where:

V_a = Injection volume of analytical column

d_p = Internal diameter of preparative column

d_a = Internal diameter of analytical column

Conditions for 30 x 150 mm column:

- Flow rate: 42 mL/min
- Injection volume: 3,000 µL

Make sure the flow rate for your preparative column can be delivered by your instrument.

Step 6: Scaling: The Importance of Dwell Time/Volume

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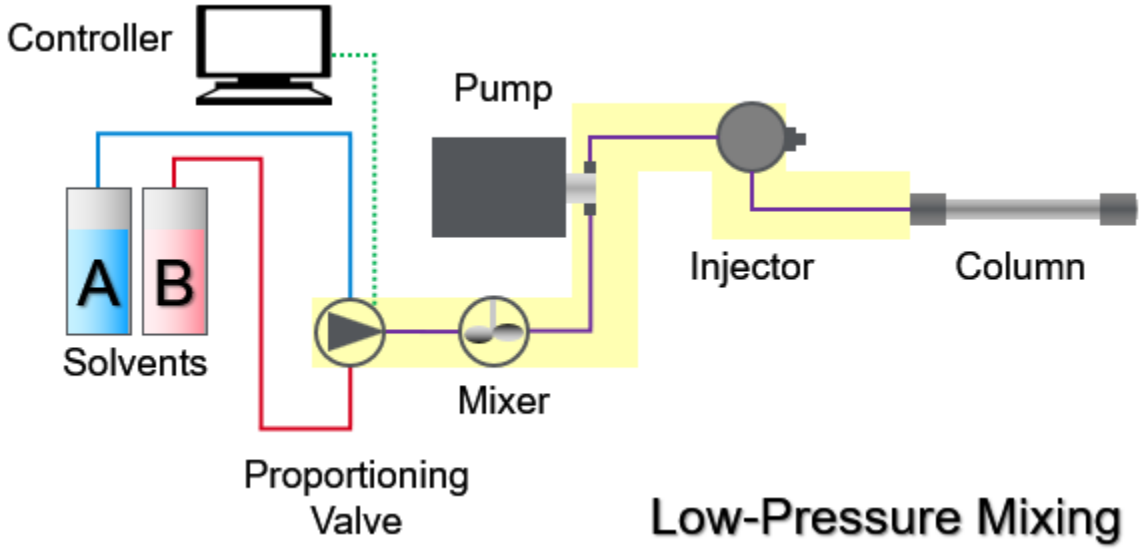
1. Check sample formulation | 2. Screen columns | 3. Optimize separation | 4. Determine max sample load | 5. Determine prep column size | **6. Scale conditions to prep column** | 7. Purify the compounds



https://community.agilent.com/technical/consumables/w/wiki/2897/lc-method-translation---the-dwell-volume

- **Dwell time (yellow path):** the time it takes for the mobile phase to travel from the mixing point in the pump to the head of the column
- **Affected by:** type of pump, autosampler, tubing diameter, and length
- **Can cause:** changes in retention time and resolution

- Difference in dwell time was calculated to 0.8 min
- Added as an isocratic hold to the beginning of the purification method

Want more info? Visit: http://explore.agilent.com/preparative-lc-primer

Step 7: Purify the compound

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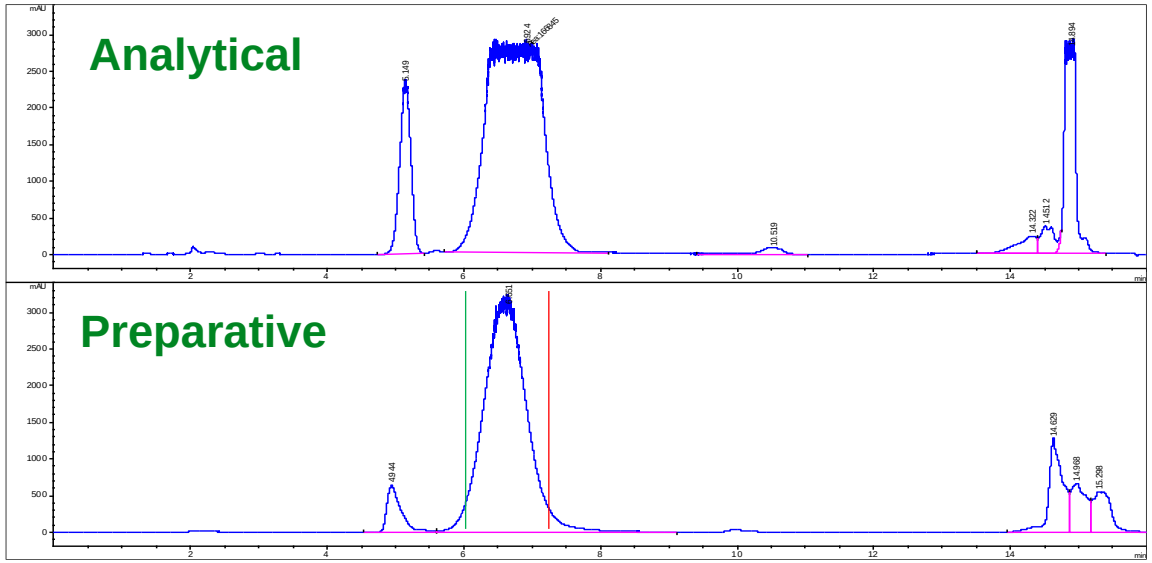
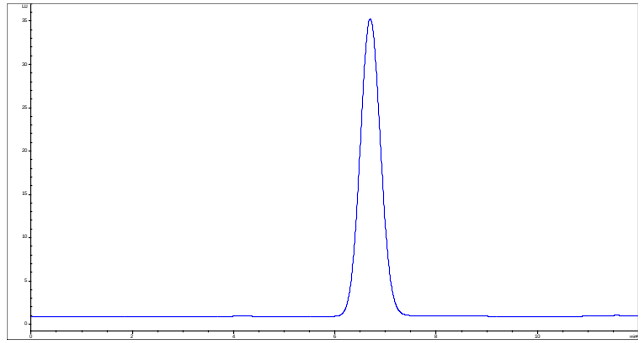
1. Check sample formulation | 2. Screen columns | 3. Optimize separation | 4. Determine max sample load | 5. Determine prep column size | 6. Scale conditions to prep column | **7. Purify the compounds**

- Since the sample is well understood, UV detection is typically all that's required
- Samples are pooled – all collections of the same compound are deposited into the same container
- Collections are triggered by a combination of threshold and time windows to minimize collection volume
- Collection is reinjected onto analytical column to verify purity



Reinject

Collection



One peak = confirms purity

Tip

Nervous to make that first big preparative injection? Make a small one first to check that your scale up calculations are correct.

High-Throughput Purification

Need For a High-Throughput Environment

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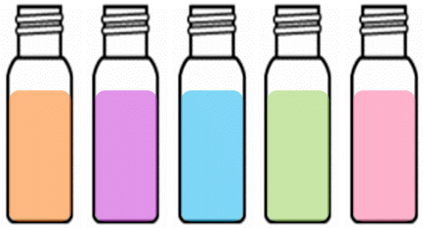
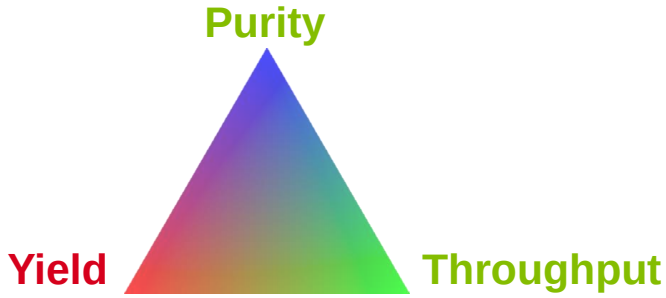
High throughput





What's important?

- Collecting enough material (10 to 100 mg)
- High purity
- Minimal method development

Ways to improve throughput:

- Screening (step 2) – analytical columns
- Optimizing (step 3) – focused gradients

Step 2: Improving Screening by Leveraging L/d_p

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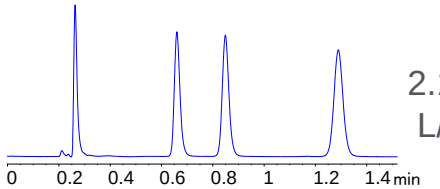
1. Check sample formulation | **2. Screen column phases** | 3. Optimize separation | 4. Determine max sample load | 5. Determine prep column size | 6. Scale conditions to prep column | 7. Purify the compounds

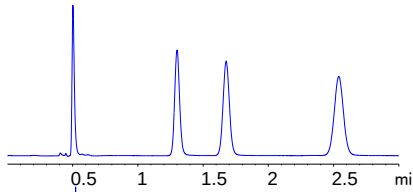
- Preferred columns: 2.1 x 50 mm, sub-2 µm or smallest available to further improve throughput
- Small columns and fast gradients (2 to 3 min) are combined to minimize method development time
- Methods can be aggressive – the goal is to identify a column/mobile phase combination that will have the highest chance of separation, not a final separation
- Columns with close L/d_p have similar resolving power

Screen with sub-2 µm columns and then scale with L/dp (length/particle diameter)

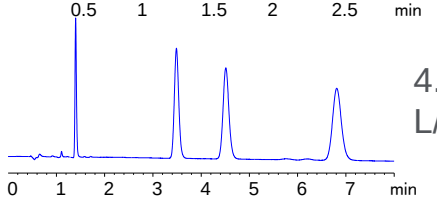
UHPLC



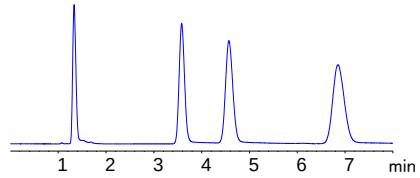
2.1 × 50 mm, 1.8 µm
L/d_p = 50/1.8 = 27.8 — **1.4 min**



2.1 x 100, 3.5 µm
L/d_p = 100/3.5 = 28.6 — **2.5 min**



4.6 x 150, 5 µm
L/d_p = 150/5 = 30 — **7.0 min**



21.2 x 150, 5 µm
L/d_p = 150/5 = 30 — **7.0 min**

Preparative

Step 3: Optimize Separation (High-Throughput Purification)

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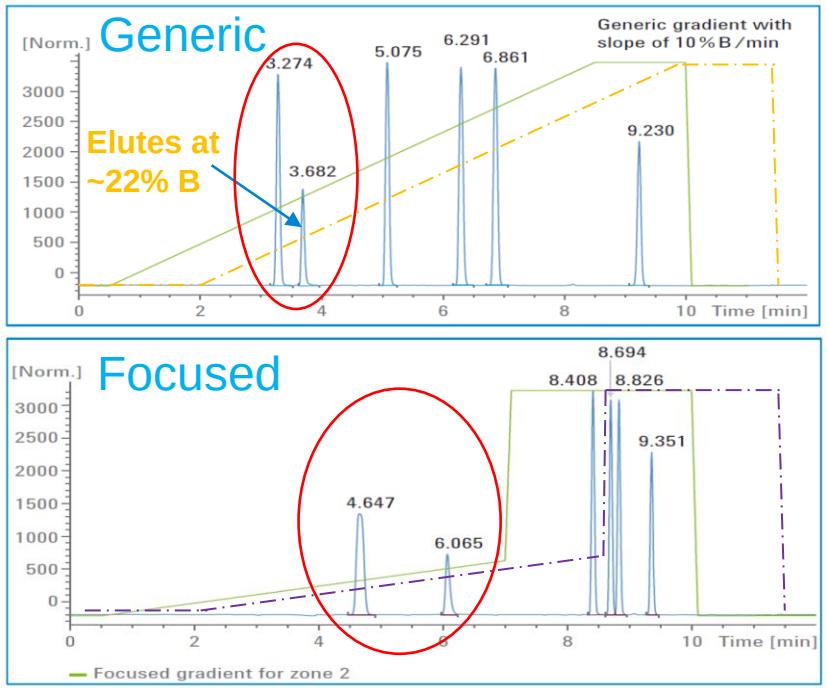
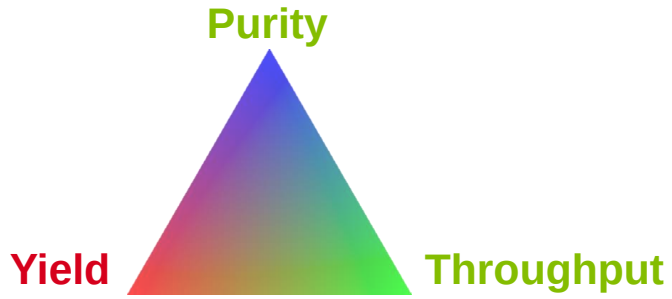
1. Check sample formulation
2. Screen column phases
3. Optimize separation
4. Determine max sample load
5. Determine prep column size
6. Scale conditions to prep column
7. Purify the compounds

- Running a focused gradient can tremendously improve the critical pair's separation with minimal method development
- Focused gradient – reducing the slope of the original gradient specific to the peaks of interest



Generic gradient (adjusted):
5% B: 0 to 2.0 min
85% B: 10.0 min
85% B: 10 to 11.5 min
Slope: 10% B/min

Focused gradient (adjusted):
5% B: 0 to 2.0 min
25% B: 8.5 min
85% B: 8.6 to 11.6 min
Slope: 3.1% B/min



Want more info? Visit http://explore.agilent.com/preparative-lc-primer

Steps 4 and 5: High-Throughput Purification

1. Check sample formulation
2. Screen column phases
3. Optimize separation
4. Determine max sample load
5. Determine prep column size
6. Scale conditions to prep column
7. Purify the compounds

4. Determine max sample load

- Generally not done for high-throughput work since only a small amount of sample is required

5. Determine preparative column size

- Instead, a column is chosen based on the anticipated load. The most popular column ids for discovery work are 21.2 and 30 mm id.

Column ID	Flow Rate	Difficult Separation (1.2 < α < 1.5)
21.2 mm	20 mL/min	20 – 70 mg
30 mm	40 mL/min	40 – 140 mg

Steps 6 and 7: High-Throughput Purification

Agilent InfinityLab

1. Check sample formulation | 2. Screen column phases | 3. Optimize separation | 4. Determine max sample load | 5. Determine prep column size | **6. Scale conditions to prep column** | **7. Purify the compounds**

6. Scale conditions to preparative column

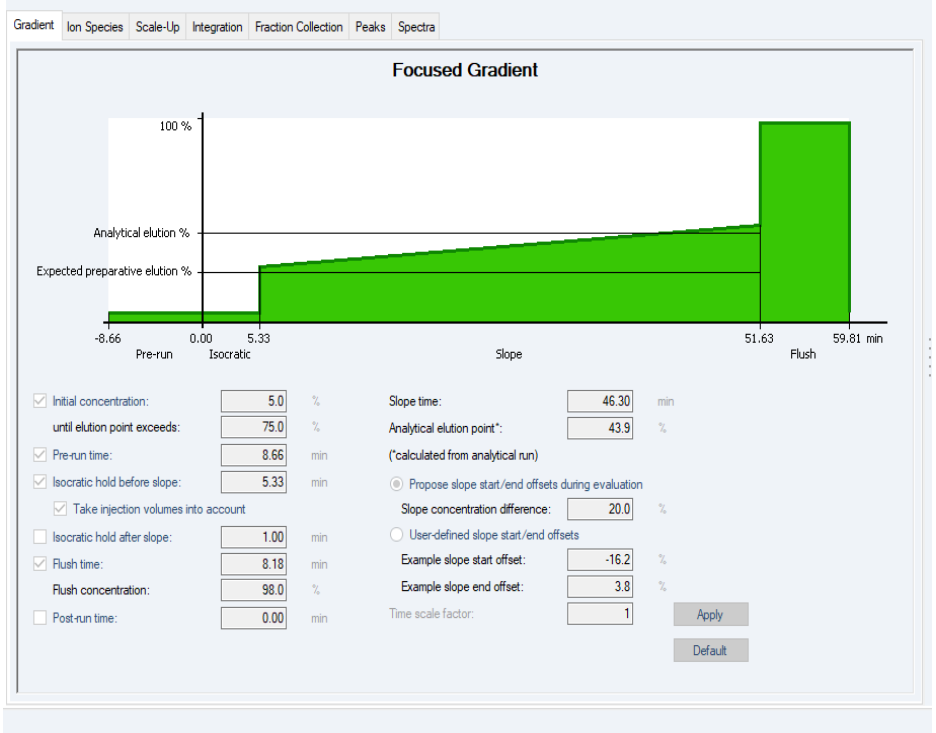
- Premade focused gradients
- A generic gradient of 10-50% B would have four focused gradients:
  10 to 20%, 20.1 to 30%, 30.1 to 40%, 40.1 to 50%
- User can select gradient based on peak's elution percentage

Manual calculation

$$f_p = f_a \left(\frac{d_p}{d_a}\right)^2, \; V_p = V_a \left(\frac{d_p}{d_a}\right)^2$$

f_a = Analytical flow rate
d_p = Prep internal diameter
d_a = Analytical internal diameter
V_a = Analytical injection volume

Automated calculation



7. Purify the compounds

- Generic trigger settings to accommodate most collections
- Always a clean waste bottle in case a compound is missed
- MS detection is typically used to minimize number of collections made

InfinityLab Preparative HPLC Columns

Family	Phase	21.2 mm ID				30 mm ID			
		50	100	150	250	50	100	150	250
Poroshell	SB-C18	670050-902	--	670150-902	--	--	--	--	--
	HPH-C18	670050-702	--	670150-702	--	--	--	--	--
ZORBAX	Eclipse Plus C18	595050-902	595100-902	595150-902	595250-902	575050-902	575100-902	575150-902	575250-902
	Eclipse Plus C8	595050-906	595100-906	595150-906	595250-906	575050-906	575100-906	575150-906	575250-906
	SB-C18	585050-902	585100-902	585150-902	585250-902	565050-902	565100-902	565150-902	565250-902
	SB-C8	585050-906	585100-906	585150-906	585250-906	565050-906	565100-906	565150-906	565250-906
	EP Phenyl-Hexyl	595050-912	595100-912	595150-912	595250-912	575050-912	575100-912	575150-912	575250-912
Pursuit XRs	C18	INF6000050X212	INF6000100X212	INF6000150X212	INF6000250X212	INF6000050X300	INF6000100X300	INF6000150X300	INF6000250X300
	C8	INF6010050X212	INF6010100X212	INF6010150X212	INF6010250X212	INF6010050X300	INF6010100X300	INF6010150X300	INF6010250X300
	Diphenyl	INF6020050X212	INF6020100X212	INF6020150X212	INF6020250X212	INF6020050X300	INF6020100X300	INF6020150X300	INF6020250X300

Phase	Surface Area (m^2/g)	Pore Size (Å)
Poroshell SB-C18 (4 µm SPP)	130	120
Pursuit XRs C18 (5 µm TPP)	440	100

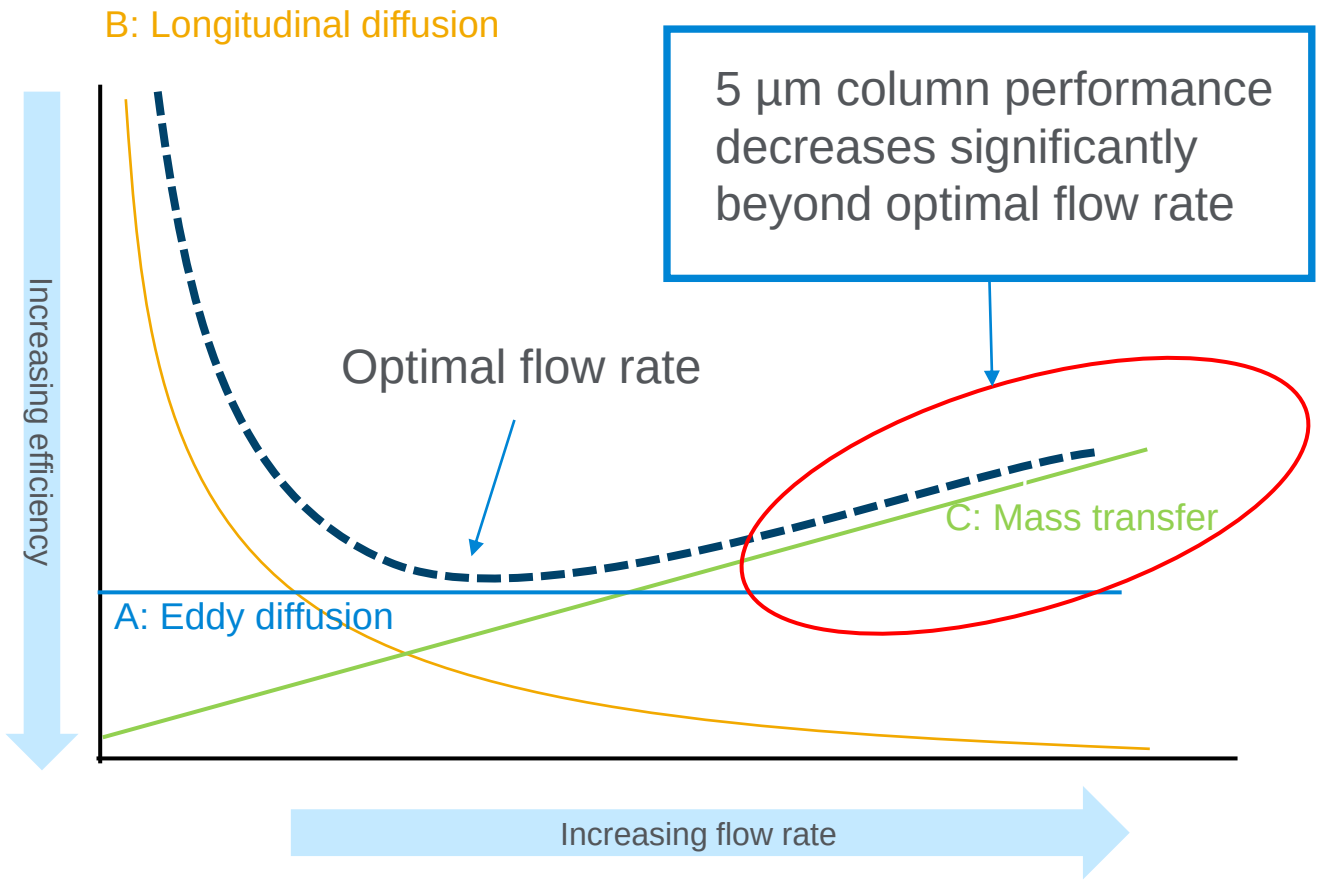
Van Deemter Equation – Significance of A, B and C Terms

$$H = A + \frac{B}{u} + Cu$$

Where:

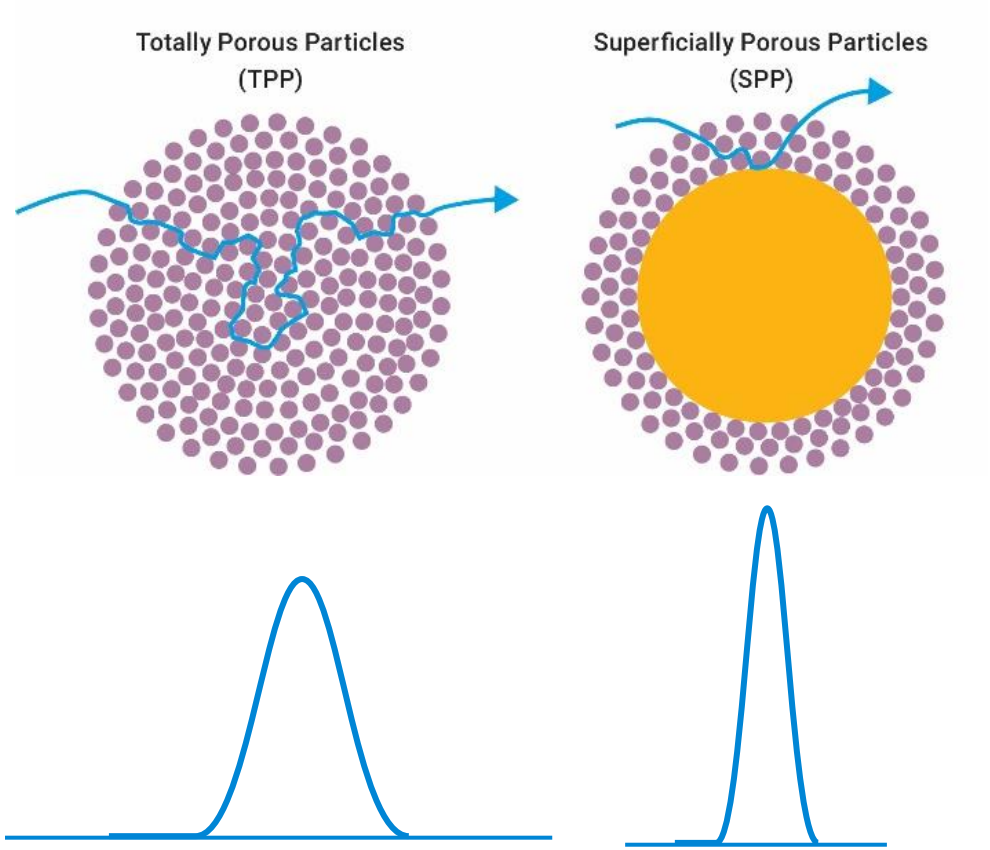
- **A term**: eddy diffusion
  - Flow path of analyte through stationary phase particles
  - Particle size, size distribution, and packing quality
- **B term:** longitudinal diffusion
  - Diffusion in the mobile phase
  - Only significant at very low flow rates
- **C term:** mass transfer resistance
  - Analyte traveling in/out of particle
  - **Significant at mid-to-high flow rates**

Van Deemter plot for 5 µm TPP column

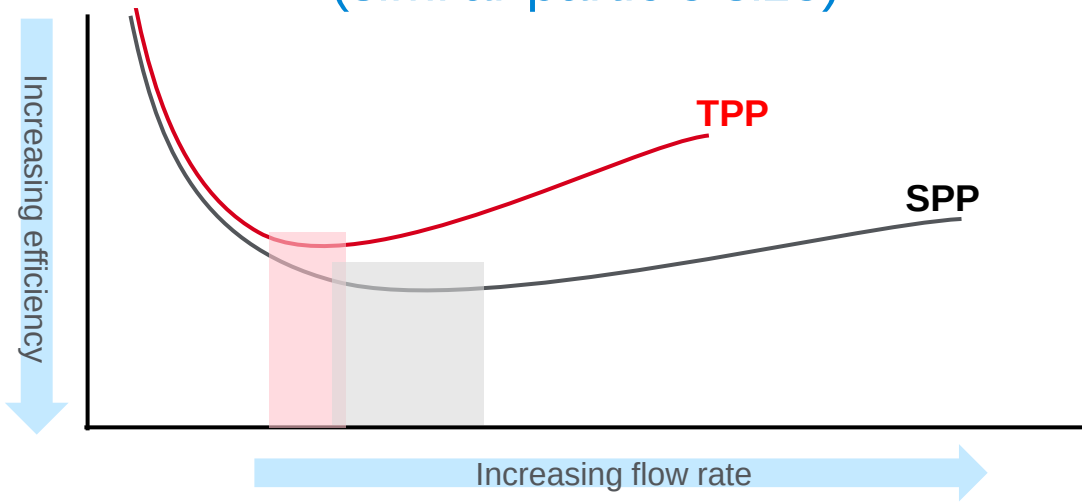


Superficially Porous Particles

- Superficially porous particle (SPP) – solid core and porous outer layer
- Solid core shortens diffusion path, resulting in a narrower chromatographic peak over totally porous particle (TPP) column



Van Deemter plots for SPP and TPP columns (similar particle size)



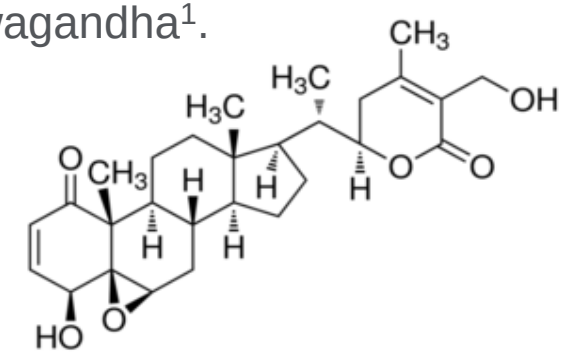
- SPP columns have smaller C terms
- Can run them at higher flow rates while maintaining column efficiency

Generic Purification Methods on SPP and TPP Columns

Separation of withaferin A in ashwagandha

- Discovery labs regularly purify large batches of samples.
- They require small amounts (10 to 100 mg) of high purity fractions for downstream workup and characterization
- Purification of bioactive components in natural products represents a similar challenge to that of drug candidates in crude mixtures – both have complicated matrices.
- Withaferin A (WFA) is the most bioactive withanolide in ashwagandha[1].

Withaferin A[2]



General Run Conditions	
Instrument	Agilent 1290 Infinity II autoscale preparative LC system
Sample	Ashwagandha extract in 2:1 ethanol:water, 100 mg/mL
Mobile Phase	A: Water + 0.1% formic acid
	B: Acetonitrile + 0.1% formic acid
Injection Volume	1 mL filtered extract

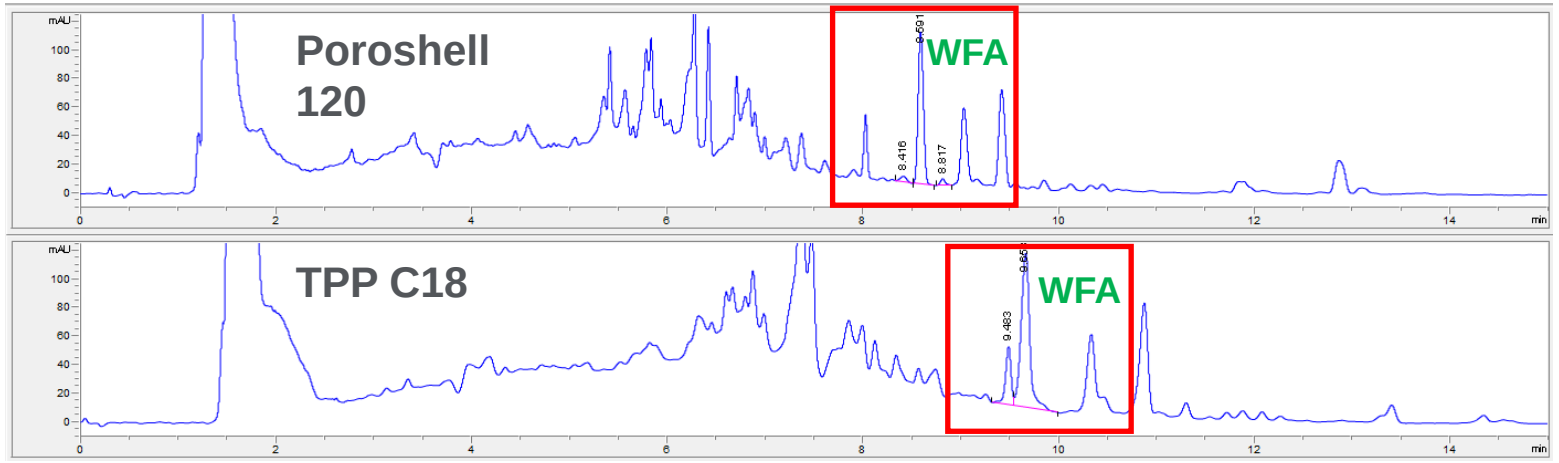
	Optimized	Elevated
Agilent InfinityLab Poroshell 120 SB-C18 21.2 x 50, 4 µm		
Flow rate:	25 mL/min	37.5 mL/min
Gradient:	5 to 95% B in 15 min	5 to 95% B in 10 min
TPP C18, 19 x 150, 5 µm		
Flow rate:	17 mL/min	25.5 mL/min
Gradient:	5 to 95% B in 18 min	5 to 95% B in 12 min

Application note: 5994-3518EN

1. M. H. Mirjalili et al. *Acta Chromatographica 25(**2013**)4, 745-754*
2. *https://www.sigmaaldrich.com/content/dam/sigma-aldrich/structure3/198/mfcd10687098.eps/_jcr_content/renditions/mfcd10687098-medium.png*

WFA Separation at Optimal and 1.5x Optimal Flow Rate

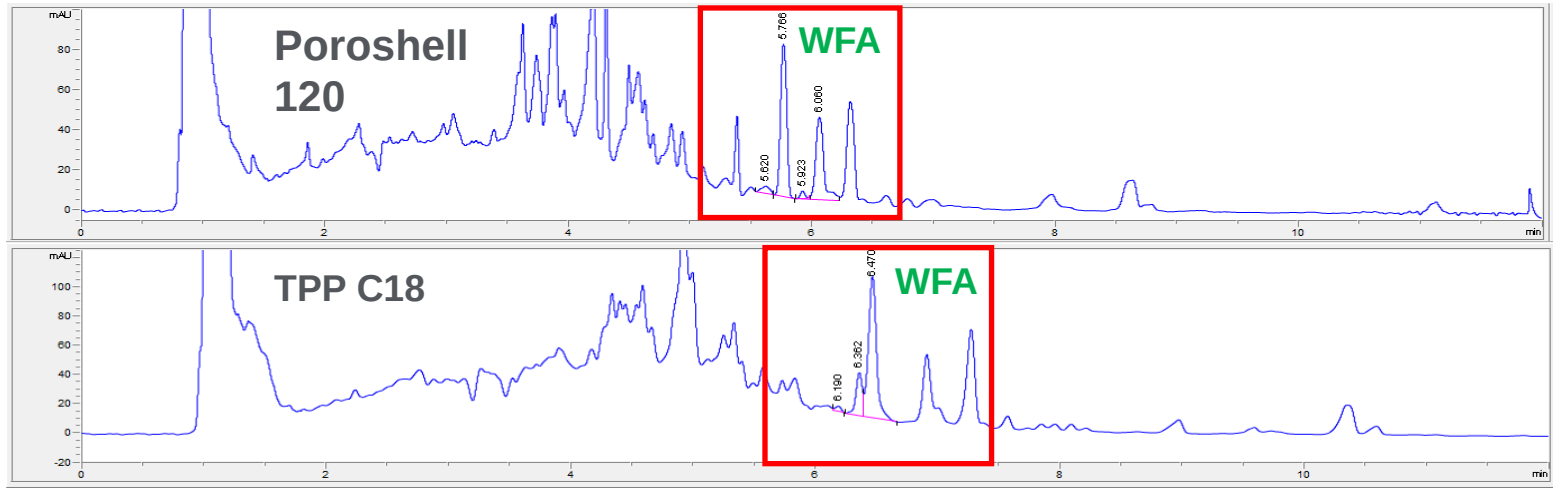
Optimal flow rate



- Separates WFA from impurities
- Best overall resolution

- Separates WFA from leading impurity
- Trailing impurity elutes in WFA tail

1.5x optimal flow rate



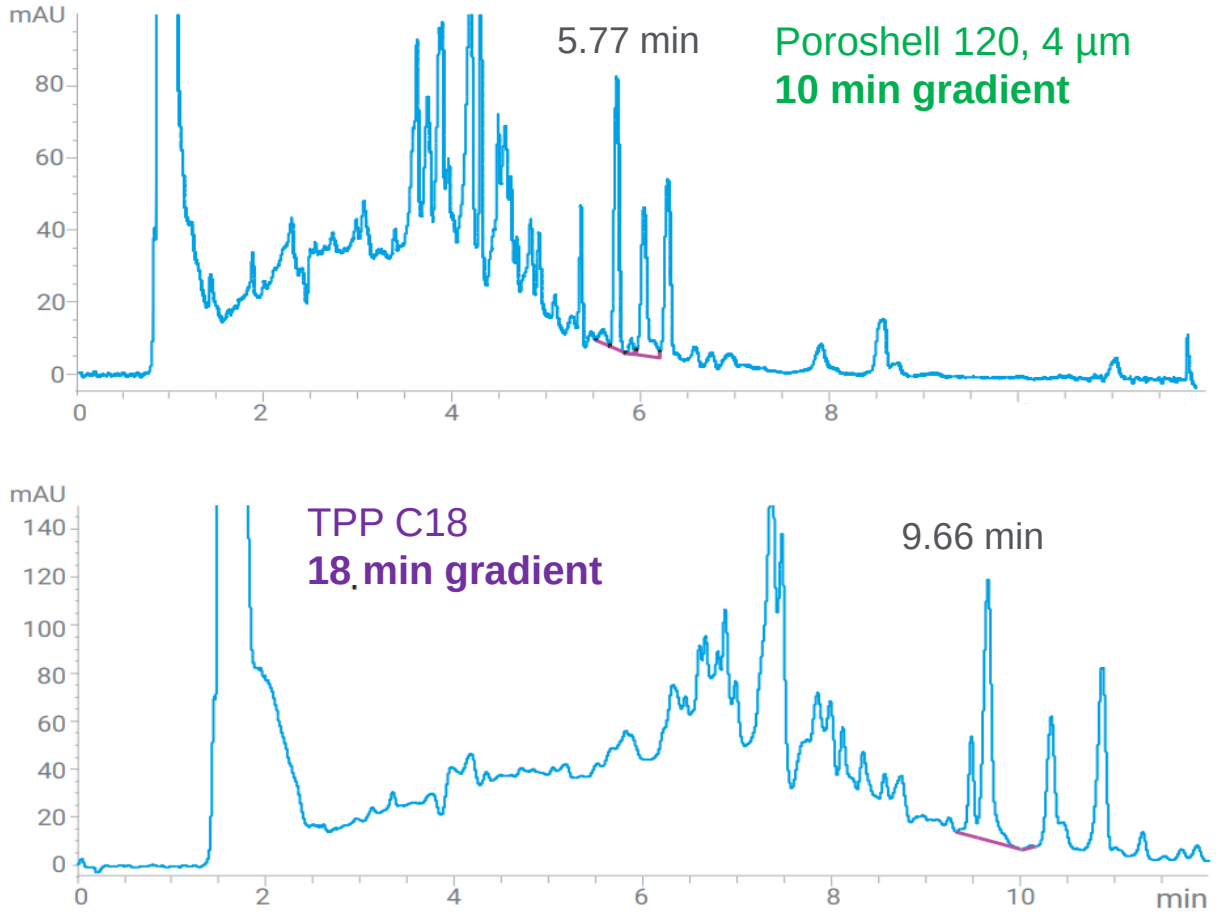
- Still separates WFA from impurities
- Best overall resolution

- Impurities have almost completely coeluted

Comparing Best Methods

1.5x optimal SPP flow rate vs optimal TPP flow rate

- SPP column maintains separation at high flow rate using a generic gradient
- TPP column loses resolution between optimal and high flow rates
- The SPP method is 45% faster than the TPP method



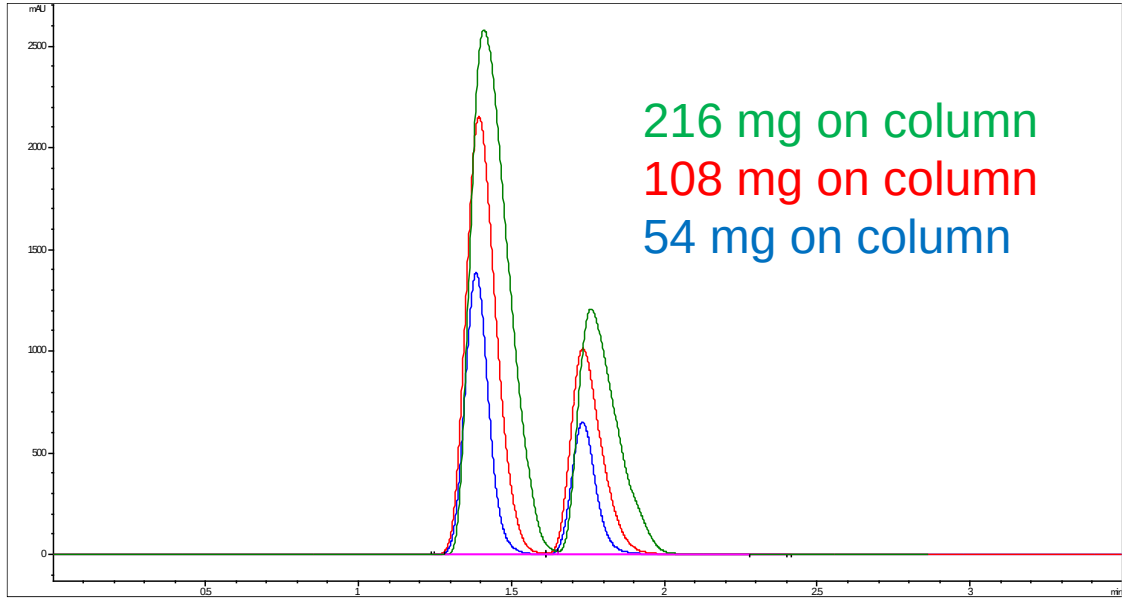
Loading on InfinityLab Poroshell 120 SB-C18

Sample: Sulfanilamide (A) + sulfamethoxazole (B) in 50/50 acetonitrile/water

- Sample 1: 30 mg/mL A + 30 mg/mL B
- Sample 2: 60 mg/mL A + 60 mg/mL B
- Sample 3: 120 mg/mL A + 120 mg/mL B

Preparative instrument	Agilent 1290 Infinity II preparative LC system
Preparative column	InfinityLab Poroshell 120 SB-C18, 21.2 x 150 mm, 4 µm
Flow rate	25 mL/min
Mobile phase	55/45 acetonitrile+ 0.1 formic acid/water + 0.1% formic acid
Injection volume	900 µL
Wavelength	238 nm

Discovery labs typically purify between 10 to 100 mg at a time



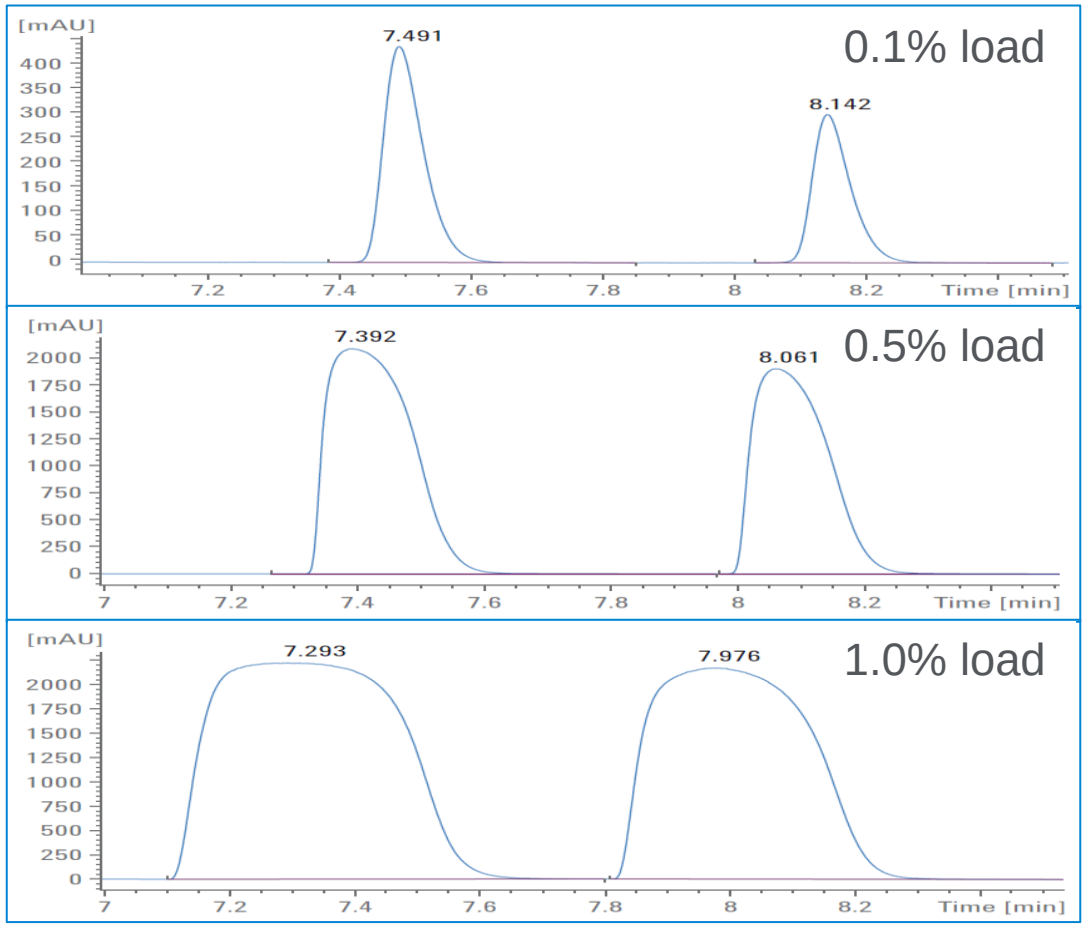
Peaks still retain shape with over 200 mg on column

Column Dimensions and Sample Load

How can we balance throughput and loading?

Column Dimensions	Injection Volume [µL]	Stationary Phase [g] with Density 0.6 g/mL	Column Load [mg]		
			0.1 %	0.5 %	1.0 %
2.1 x 50 mm, C18	1.75				
4.6 x 150 mm, C18	25				
19 x 100 mm, C18	284	17	17	85	170
19 x 150 mm, C18	426	26	26	130	260
19 x 250 mm, C18	710	43	43	215	430
21.2 x 100 mm, C18	354	21	21	105	210
21.2 x 150 mm, C18	531	32	32	160	320
21.2 x 250 mm, C18	885	53	53	265	530
30 x 100 mm, C18	706	42	42	210	420
30 x 150 mm, C18	1063	64	64	320	640
30 x 250 mm, C18	1772	106	106	530	1060
50 x 100 mm, C18	1969	118	118	590	1180
50 x 150 mm, C18	2953	177	177	885	1770
50 x 250 mm, C18	4922	295	295	1475	2950

- Data based on lab experiments
- DMSO as the sample solvent
- Column loads depend on separation being easy (1%), average (0.5%) or difficult (0.1%)



Preparative LC Scaling Calculator

Agilent InfinityLab

Preparative LC Scaling Calculator | Agilent

	Analytical Method	Preparative Method
System Description		
System name		
Minimum flow rate (mL/min)		
Maximum flow rate (mL/min)		
Dwell volume (mL)		
Injector cycle time (min)		
Solvent A		
Solvent B		

Analytical Gradient				Preparative Gradient			
Time (min)	Flow (mL/min)	%A	%B	Time (min)	Flow (mL/min)	%A	%B
0				0			

Add row

Column Information			
Phase			
Particle size (µm)*			
ID (mm)*			
Length (mm)*			
Flow rate (mL/min)*			
Injection volume (µL)			
Compound concentration (mg/mL)			
Override preparative flow rate	Yes / No		
Desired amount of purified compound (mg)			
Current mass on column (mg)			
Estimated column capacity (mg)			
Column void volume (mL)			

Oligonucleotide Purification

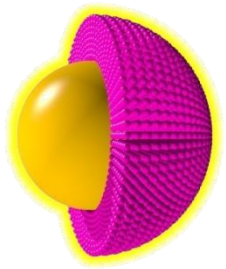
Ion-pair reverse phase

Particle Support and Column Chemistry

Reversed Phase Column Options for Oligonucleotides

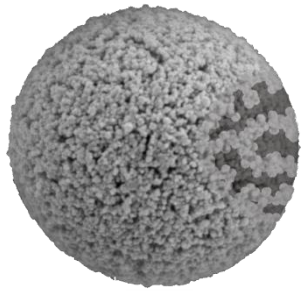
AdvanceBio Oligonucleotide

- Fully scalable column chemistry platform
- Available in analytical dimensions through 21.2 mm id preparative
- Analytical characterization post-purification
- High efficiency, 2.7 and 4 µm superficially porous particle, 120Å pore
- Silica-based C18 with novel, high pH-resistant modification
- UHPLC resolution at HPLC pressure



PLRP-S

- Scalable for purification, including bulk media
- Variety of pore sizes for all sizes and types of oligos – 100Å, 300Å, 1000Å, 4000Å
- Inherently hydrophobic surface (no bonded phase alkyl ligand required for reversed-phase separations, so no ligand leaching)
- Polymeric, stable up to pH 13
- High binding capacity



IP-RP: Method Development Optimizations

Agilent InfinityLab

Common variables to consider:

- **Detector**
  - 100 mM TEAA/water-acetonitrile gradient — UV method
  - TEA + HFIP/water-methanol gradient — MS method
  - (TEAA reduces signal response of target compound)

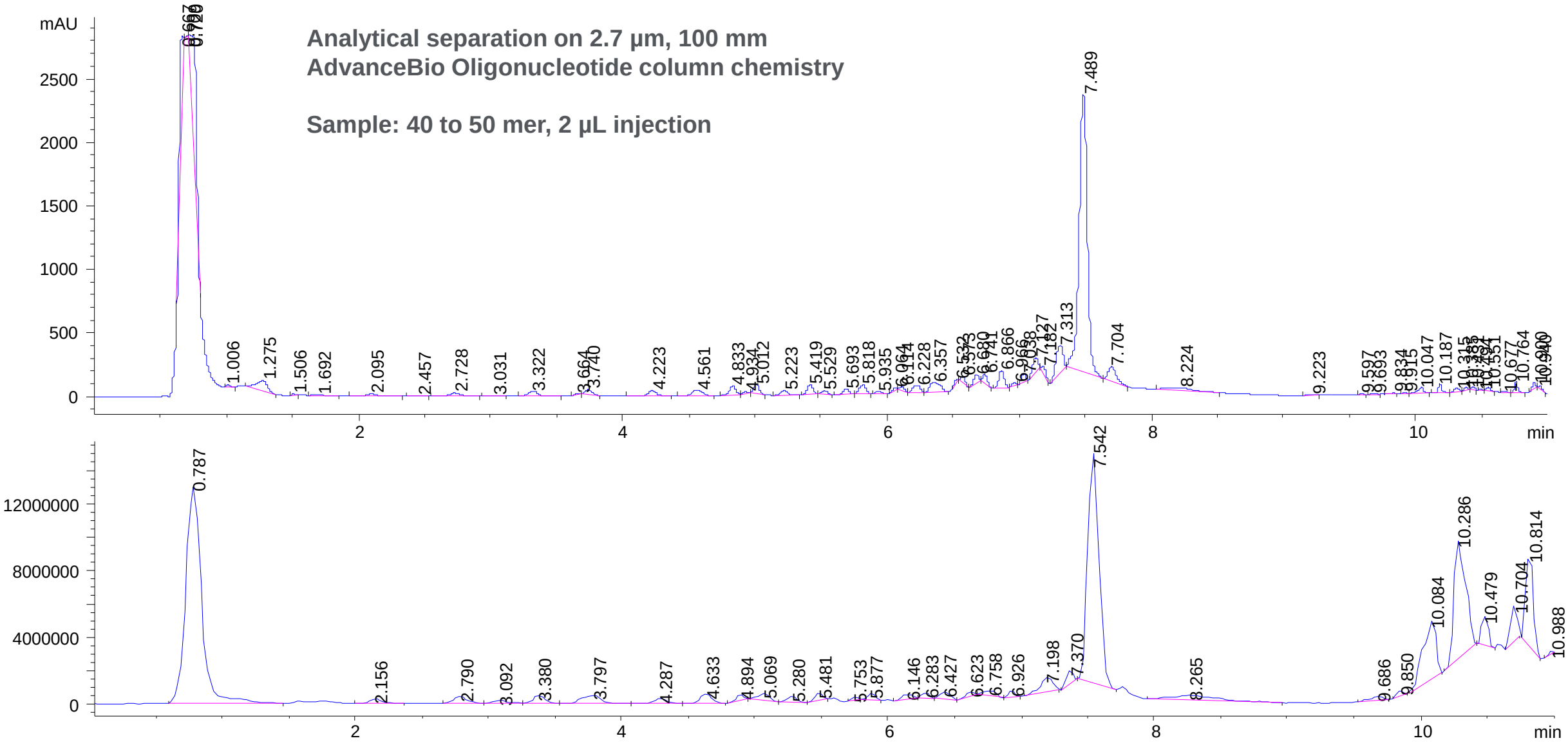
- **Ion-pairing agent selection**
  - Mobile phase composition (IP agent and concentration) affects MS sensitivity and mobile phase pH

- **Temperature**
  - Elevated temperatures are used to denature the oligo and reduce secondary interactions
  - 50 – 60°C is commonly used

- **Particle support and column chemistry**
  - Optimal separation conditions require particle to be stabile under high pH and elevated temperatures
  - SPP offers increased resolution at high flow rates and lower back pressures
  - RP column chemistry (C18, C8, diphenyl) affects retention and separation of target compound from closely related impurities

Analytical Characterization to Preparative Purification for Oligonucleotides

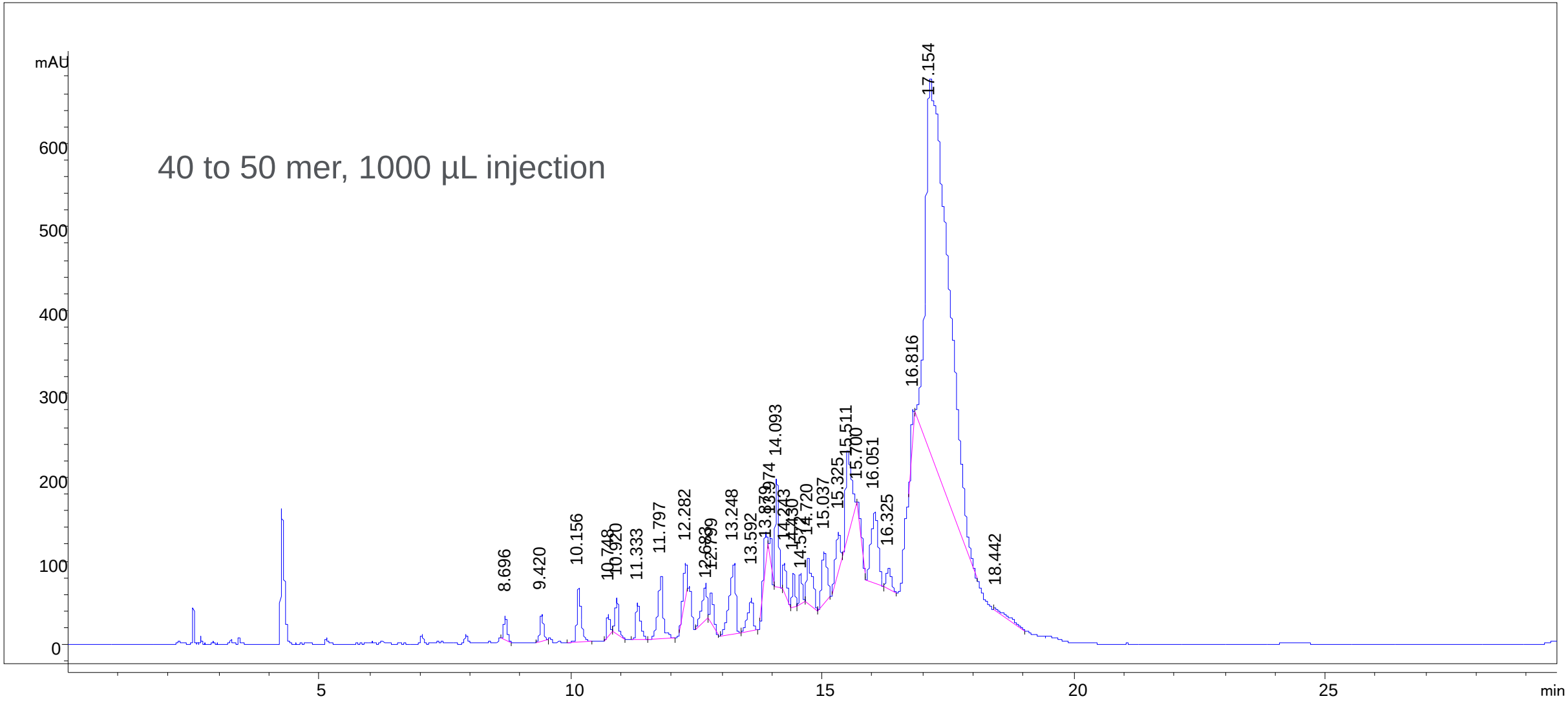
Agilent InfinityLab



Agilent

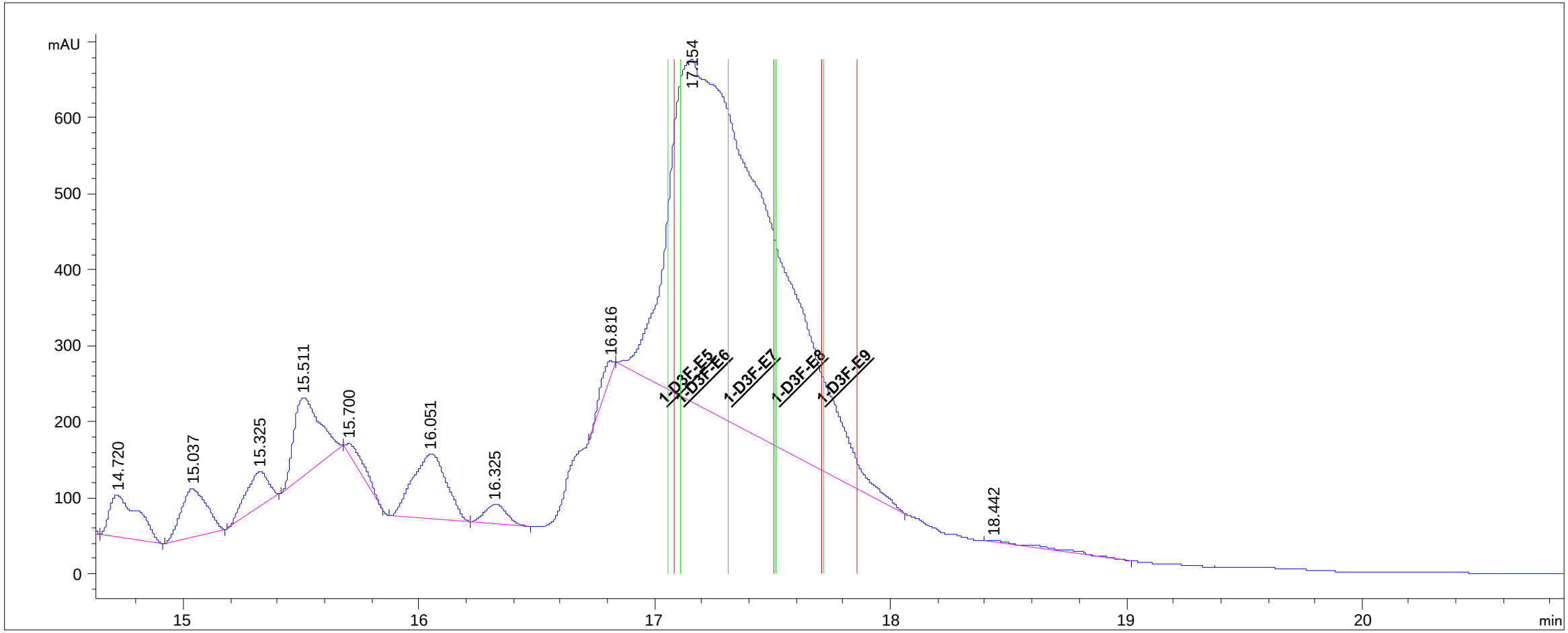
Preparative Purification of Oligonucleotides

AdvanceBio Oligonucleotide 21.2 × 150 mm, 4 µm



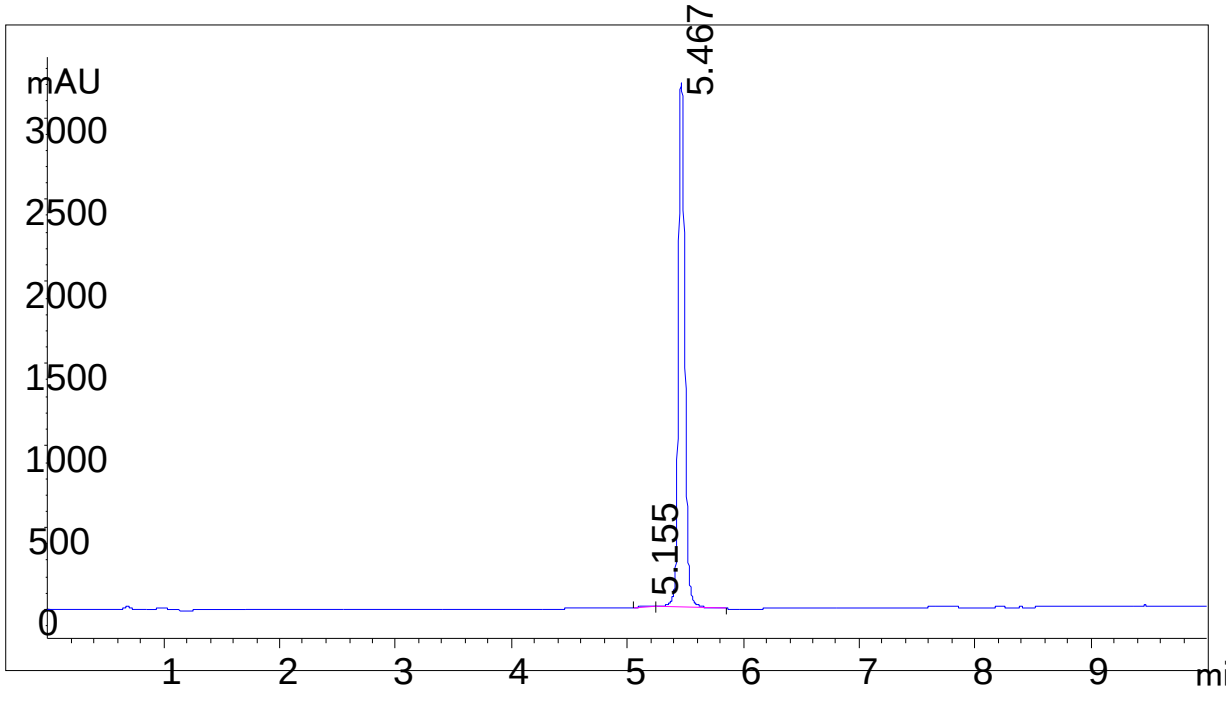
Purification of Oligonucleotides

AdvanceBio Oligonucleotide 21.2 × 150 mm, 4 µm

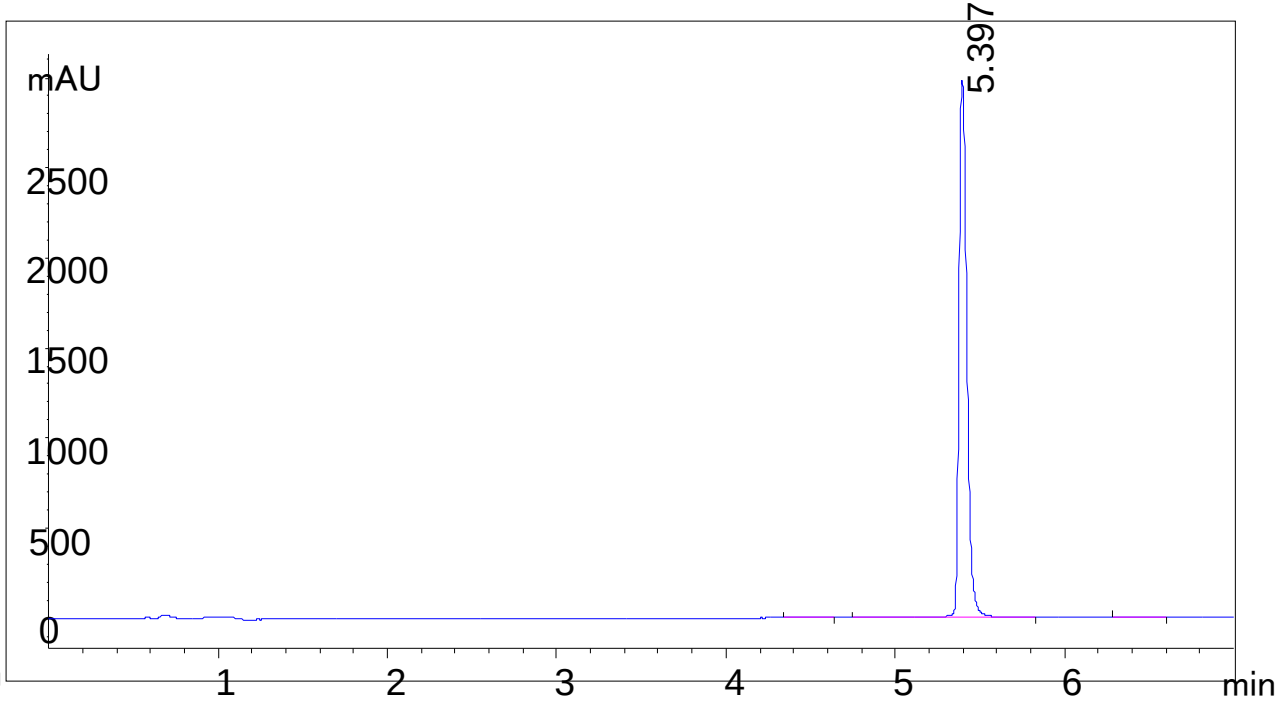


Analytical Characterization after Purification

Fraction reanalysis



fraction #1: 99.5%



fraction #2: 99.7%

Matching Columns to Instruments

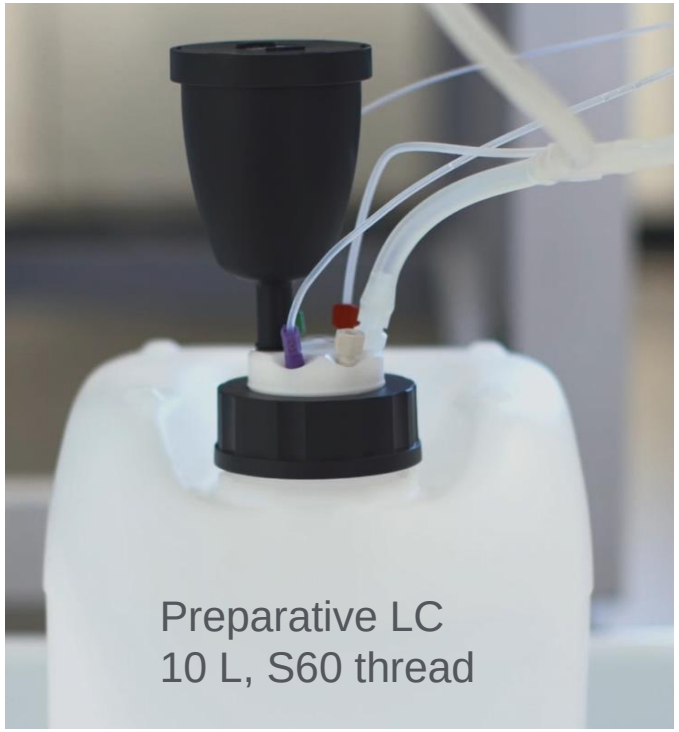
Agilent InfinityLab

Column ID	Analytical	Semi-Preparative	Preparative
2.1 mm	0.1 – 0.2 mL/min		
4.6 mm	0.5 – 1.0 mL/min		
7.5 to 10 mm		1.5 – 5.0 mL/min	
21.2 to 25 mm			15 – 30 mL/min
50 mm			60 – 120 mL/min
100 mm			240 – 480 mL/min
	1220/1260/1290 Infinity II Analytical-scale LC Purification System [0.1 mL/min – 10 mL/min]		
		1260 Infinity II Preparative LC System [1 mL/min – 50 mL/min]	
		1290 Infinity II Preparative LC System [1 mL/min – 50 mL/min]	1290 Infinity II Preparative LC System [4 mL/min – 200 mL/min]

InfinityLab Supplies Designed for Preparative LC

With prep comes more solvent

Larger volumes need careful containment

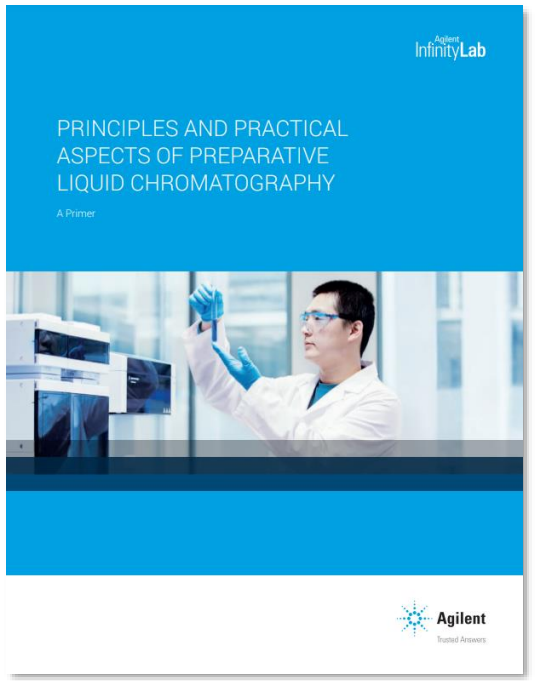


- ✓ Tubing Kits
- ✓ Sample loops
- ✓ Trays for fraction collector
- ✓ Sample containment, test tubes

Quick Reference Guide for Prep Supplies : 5994-2810EN
Stay Safe Caps flyer: 5991-5162EN

Resources for support

- **Product webpage:** www.agilent.com/chem/prepcolumns
- **Product brochure:** InfinityLab Poroshell120 Preparative LC Columns 5994-3601EN
- **Primer:** Principles and Practical Aspects of Preparative Liquid Chromatography 5994-1016EN
  - A great overview that covers fundamentals and best practices
- **Application note:** A Tale of Two Samples
  - Part 1: Bulk Purification 5994-4707EN
  - Part 2: High-throughput Purification 5994-4708EN
- **Oligonucleotide resources**: Agilent Oligonucleotide Chromatography Solutions | Agilent



Agilent InfinityLab